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16. Abstract

The feasibility of producing antibodies to DDT and Malathion derivatives was accomplished. ~~This was accomplished~~ by immunizing animals with pesticide-protein complexes in which the protein carrier was derived from the species being immunized. DDA-rabbit serum albumin or Malathion anhydride-rabbit serum albumin conjugates were injected into rabbits in complete Freund's adjuvants at multiple intradermal sites, and antibody responses were encountered by the third or fourth injection. The results demonstrate that (1) it is possible to develop specific antibodies to two pesticidesderivatives, DDA and Malathion anhydride (2) these antibodies are highly specific for each pesticide derivative; and (3) given enough time, the technical difficulties which have been encountered in utilizing these antibodies for the development of a highly sensitive immunoassay procedure could be resolved. Such a technique would offer investigators interested in pollution problems a simultaneously highly specific and highly sensitive method for detecting and measuring these pesticides, without the need for preliminary treatment of the samples being investigated.

17a. Descriptors *DDT, *Malathion, *Pesticides, *Antibiotics (Pesticides), ~~Water pollution effect~~ Public health, Pollutant identification

17b. Identifiers DDA, *Immunization, Immunoassay

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describing work performed under
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"WATER MONITORING SYSTEM"

Period covered June 24, 1968 - December 23, 1969

Cordis Laboratories
2140 North Miami Avenue
Miami, Florida 33127

March 3, 1970

INTRODUCTION

The rapidly increasing pollution by man of his environment is currently creating extremely serious problems on a global scale. One facet of this pollution involves the ever-widening use of pesticides, herbicides, and similar substances. These can sometimes pose threats of direct cumulative toxicity, as in the repeated consumption of foodstuffs containing small quantities of them. They also can seriously affect the ecological balance and produce insidious disruptions of the food cycle. This results from the tendency of organisms in a food chain to sequentially concentrate many of the nonbiodegradable chemicals in their tissues as they feed on other organisms containing only low concentrations of them. This phenomenon, called "biological magnification", has been implicated in the poisoning of fish-eating birds, for example. One study of a river estuary in New York revealed that the water itself contained only 0.05 parts per billion (PPB) of DDT residues. However, the plankton there contained 40 PPB: shrimp, 160 PPB; clams, 420 PPB; flounder, 1,280 PPB; herring gull, 9,600 PPB; cormorant, 26,400 PPB; and ringed bill gull, 75,550 PPB. The concentrations in some of these species approached or achieved known toxic levels.

The spreading nature of this problem is indicated by the fact that species which exist far from civilization also appear to be getting contaminated with such pesticide residues. For example, chicks of the Bermuda petrel, which spends most of its life ranging the open

sea at great distances from applications of DDT, have been found to contain up to 7,000 PPB of DDT residues. These levels are known to produce significant toxic effects in related species.

Such accumulations of these nonbiodegradable toxic chemicals could also readily involve man as he consumes foods so tainted below him in the ecosystem. It is abundantly clear that sensitive and precise detection and measuring systems are required to monitor the levels of these contaminations at the source, e.g., in water reservoirs and streams.

Although considerable success has been obtained in detecting and measuring pesticide residues by gas chromatography, even more sensitive and specific methods would be highly desirable. One possible approach involves the use of immunochemical techniques, since extraordinary successes have been recently achieved in the measurement of antigenic substances by specific immunological reagents and techniques. For example, as little as 0.000,000,001 mg/ml of several pituitary hormones can be directly detected by such approaches (equivalent to 0.000,001 parts per million). Since recent reports have indicated that specific antibodies to two widely used pesticides can be produced in experimental animals, it was felt that similar immunoassay methods should be applicable to the detection and measurement of equivalently minute trace quantities of these pesticides. The research effort by the Cordis Laboratories, supported by Contract #14-12-412 from the Department of Interior, was thus aimed (1) at the production of specific antibodies to DDT and Malathion derivatives and (2) at

the application of these antibodies to the development of isotope immunoassay procedures for these pesticides. The ultimate goal of this program is to develop similar procedures for the continuous monitoring of such pesticides in water supplies or water souces.

B A C K G R O U N D

Rabbit antisera to both DDT and Malathion derivatives have now been reported by two groups of investigators. In both instances (Centeno and Johnson, Fed. Proc., 1967, 26:2497; Haas and Guardia, Proc. Soc. Exptl. Biol. & Med., 1968, 129:546) the antibodies were formed against covalently bonded conjugates of these pesticides with certain heterologous protein carriers. They were reported to be specific for the pesticides used and not to show cross-reactions with each other. In the study by Haas and Guardia, no antibody responses were obtained following immunization of rabbits with the pesticide residues bound to rabbit serum albumin (RSA) or bovine serum albumin (BSA). However, the same pesticide residues coupled to bovine fibrinogen elicited antibody responses, but these were partially directed against the heterologous carriers. Suitable absorptions with the carrier (bovine fibrinogen) resulted not only in considerable decreases in titer but also in some specific residual reactions with the pesticide haptens, e.g., DDA or Malathion anhydride.

The highly sensitive immunoassays which have been developed for hormones and other antigenic substances usually depend on insolubilized but active specific antibody. The active sites compete for the substance in the test sample and in added known quantities of the isotopically labeled antigen. The amount of displacement of the latter from the insolubilized antibody gives a precise measure of the amount of the substance in the unknown sample. This principle was applied, for example, when Wide and Porath (Biochim. Biophys. Acta, 1966, 130, 257) prepared active insolubilized antibody to human pituitary luteinizing hormone by covalently bonding it to small particles of Sephadex. To these suspensions were added varying quantities of purified hormone, and time was allowed for specific binding to occur. Following this, a predetermined quantity of purified, isotopically tagged growth hormone was added to each mixture. After the reaction had taken place, the insolubilized complexes were centrifuged, washed, and counted for isotope concentration. A standard curve was thus established relating the concentration of hormone to the degree of displacement of the isotopically tagged material. The concentrations of hormone in unknown specimens could then be readily determined in relation to the standard curve.

Other techniques for preparing insolubilized active antibodies have been described and have been used in similar or related procedures. These include glutaraldehyde treatment (Immunochem.,

1969, 6, 53); covalent bonding to granular cellulose derivatives by cyanogen bromide (Biochim. Biophys. Acta, 1964, 78, 516); diazonium coupling (Immunochem., 1965, 2, 181); or more complex reactions (Biochem. J., 1966, 101, 711); cross-linking of antibody molecules to form large-molecule, insoluble aggregates via carbodiimide coupling (Science, 1965, 148, 1235); disulfide linkages (Biochem. J., 1966, 101, 717); or through azo-bonds (Nature, 1964, 204, 265); adsorption of antibody to the inner surface of plastic tubes (Science, 1967, 158, 1570); as well as the use of isothiocyanate residues on polystyrene discs (Austral. J. Exp. Biol. Med. Sci., 1967, 45, 703). Many of these techniques have been employed in the investigations conducted on this project, as described below.

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Preparation of Purified DDA Anhydride and Malathion Anhydride for Coupling to a Protein Carrier

DDA and DDA Anhydride. DDA (di-(p-chlorophenyl)acetic acid) was prepared from DDT (di-(p-chlorophenyl)-trichloro-ethane) by the method of Grummitt et al. (Org. Syn., 1955, 3, 270). One hundred grams of DDT was added to 800 ml of diethylene glycol and 126 grams of KOH in 70 ml of water. After refluxing for 6 hours at 134-137°C, the mixture was cooled, poured into 2 liters of cold water, and filtered. After treatment with activated charcoal, the filtrate was acidified with sulfuric acid and chilled. The precipitated DDA was

washed with water, further purified from boiling ethanol, and dried (M. P. 163-165°C).

To 100 grams of DDA was added 74 ml of fresh acetic anhydride. After refluxing and evaporation of volatiles in a rotary evaporator, an additional 74 ml of acetic anhydride was added, and the refluxing and evaporation were repeated. To the remaining syrup, seed crystals of DDA anhydride were added, and the mixture was evacuated for an additional 36 hours. The resultant hard crystalline mass was crushed and washed with about 500 ml of hexane. After filtration and drying, 80 grams of crude DDA anhydride remained; this was dissolved in a hot mixture of 20:80 ethanol-hexane and then was allowed to recrystallize at room temperature. This final product had a melting point of 88.5-90°C.

As a check on its identity, a small quantity of the DDA anhydride was converted to the acid (DDA) by boiling with 10% NaOH. After neutralization with HCl, the product was washed and found to have the proper melting point (163-167°C). Early difficulties were repeatedly encountered in the crystallization of the first lot of DDA anhydride, but once this was accomplished, seeding for subsequent batches proved quite effective.

Malathion Anhydride. The Malathion used for direct immunization and testing was a specially prepared 99% pure sample obtained through the courtesy of Dr. Woolford of the American Cyanamid Co.,

Princeton, New Jersey.

The Malathion anhydride was synthesized by the method of March et al. (J. Econ. Entom., 1956, 49, 185) and Centeno et al. (personal communication). To 80 ml of carefully redistilled anhydrous benzene was added 70.4 mg of phosphorus pentasulfide. The temperature was adjusted to 40°C, and 57.6 ml of methanol was added dropwise. After 3 hours, the excess solvent was removed under reduced pressure, and approximately 32 grams of the O,O-dimethyl hydrogen phosphodithiolate was recovered. To this was added 19.6 grams of maleic anhydride, and the mixture was heated at 85°C for 16 hours. The resultant reddish-brown oil, which was the anhydride of O,O-dimethyl-S(1,2-bis-carboxy)-ethyl phosphodithiolate, was used without further purification.

Preparation of Conjugates of DDA and Malathion Anhydrides with Rabbit Serum Albumin for Use as Immunizing Antigens.

DDA Anhydride-Rabbit Serum Albumin Complex. To 100 ml of crystalline rabbit serum albumin (10 mg/ml, Pentex) in 0.9% NaCl containing 0.25% Na_2CO_3 in an ice bath, a solution of DDA anhydride (250 mg/3.5 ml ethanol) was added very slowly with vigorous but not violent stirring. After 30 minutes, the mixture was thoroughly dialyzed against distilled water in the cold for 2 days. After clarification by centrifugation, the DDA-protein complex was lyophilized.

After hydrolysis, analysis of these complexes by gas chromatography indicated that approximately 10% by weight consisted of

firmly bound DDA.

Malathion Anhydride-Rabbit Serum Albumin Complex. To 100 ml of crystalline rabbit serum albumin (10 mg/ml) in 0.9% NaCl containing 0.25% Na_2CO_3 in an ice bath, 250 mg of Malathion anhydride in 2.0 ml of ethanol was added very slowly with stirring. The mixture was dialyzed for 2 days against distilled water in the cold, clarified by centrifugation, and then lyophilized.

Attempts to Produce DDT and Malathion-Rabbit Serum Albumin Complexes. To prepare materials for testing the possibility that DDT and Malathion might themselves be antigenic, these parent compounds were added to rabbit serum albumin under exactly the same conditions outlined above in preparing the DDA anhydride and Malathion anhydride-protein complexes.

Immunization of Rabbits

A series of rabbits were immunized with the various pesticide-protein mixtures or complexes by repeated intracutaneous injections at multiple sites. Ten New Zealand male rabbits were used for each anhydride-RSA conjugate, and four New Zealand male rabbits were used for each native pesticide-RSA "conjugate". The immunization schedule shown below was followed. All of the animals immunized with the anhydride-protein complexes produced antibodies after four injections in a 46 day period.

Immunization Schedule

Injection	Days	Antigen (mg)	Bleeding	Precipitating Antibodies to Anhydrides
1	0	10		
2	7	10		
-	17		1	
3	25	10		
-	31		2	±
4	39	20		
-	46		3	+
5	71	30		
-	82		4	+
6	106	40		
-	121		5	+
7	134	40		
-	144		6	+

The detection of precipitating antibodies to the pesticide derivatives was made by two-directional immunodiffusion tests performed according to the Wadsworth modification of the Ouchterlony method. Positive precipitation reactions were seen in the sera of all the animals immunized with the derivative rabbit serum albumin conjugates. Precipitating antibodies to the native pesticide-albumin "conjugates" were never seen, however.

Typical reactions between the pesticide derivatives and several of the antiserum samples are shown in Fig. 1. In the tests with DDA-RSA (Fig. 1a), "reactions of identity" were noted when antibodies to DDA-RSA were present in sera from different animals. With RSA alone, DDT-RSA "conjugate", or DDT alone, the same concentrations of the same antisera showed no detectable reactions (Figs. 1b, c, and d).

In a similar way, several antisera were shown to possess antibodies to Malathion anhydride-RSA (1 mg/ml) (Fig. 2). Here, too, no reactions were seen at these concentrations with native Malathion anhydride-RSA conjugate, RSA, or Malathion itself.

In no instance did the preimmunization sera from these animals reveal positive reactions with these antigens. Moreover, the immunoglobulin fractions from the positive antisera were the only serum fractions possessing activity for anti-DDA antibodies, as illustrated in Fig. 3.

Cross tests of the specificities of these anti-DDA-RSA, and anti-Malathion anhydride-RSA antibodies were also performed by immunodiffusion assay. Typical results are shown in Fig. 4, where it can be clearly seen that each antibody is specific for its own pesticide derivative.

In a number of instances, however, the antisera resulting from immunization with either the DDA-RSA or Malathion anhydride-RSA conjugates revealed two precipitin systems. This was especially

noticeable when the test antigens for immunodiffusion were used at higher concentrations. Typical results are shown in Fig. 5, where it may be seen that a second precipitin system even occurred in reactions with normal rabbit serum albumin alone. Although their origins are not clearly known, these contaminating antibodies could be readily removed by absorption with normal serum albumin at 10 mg/ml. The results are illustrated in Fig. 6, where it may be seen that the single immune precipitate remaining is completely specific for its own pesticide derivative.

Even more unequivocal proof that these antibodies in DDA-RSA and Malathion anhydride-RSA were distinct and specific for each hapten was demonstrated by cross-absorption studies. Thus, absorption of anti-DDA-RSA with Malathion anhydride-RSA left the reaction with DDA-RSA intact. The reverse was also true, and these findings are illustrated in Fig. 7.

These reactions of antibodies to the pesticide derivatives were also studied by immunoelectrophoresis. The specificity of each was confirmed by this technique, and, in addition, it was demonstrated that the conjugated antigens migrate differently in an electrophoretic field than do the native rabbit serum albumin carriers. Typical results are shown in Fig. 8, where the absence of cross-reactivity in the immune system is clearly apparent. The difference between the mobility of Malathion anhydride-RSA and that of RSA itself (Fig. 8e) is more striking than the change in the DDA system (Fig. 8f). However, the

formation of an immune precipitate between the goat antibodies to rabbit serum albumin and each of the conjugated hapten complexes demonstrates convincingly that this protein carrier is strongly bound to the hapten and is still reactive immunochemically.

Further data regarding the amount of hapten substitution in the albumin molecule were obtained with a sample of DDA-RDA. By using gas chromatography after digestion, it was found that the specimen contained about 10% of covalently bonded DDA.

Anti-Hapten Antibody and Normal Gamma Globulin Concentrates. These were usually prepared by salting out with ammonium sulfate at 1/3 saturation. The normal rabbit sera used were the preimmune specimens from the animals supplying the antibodies. These immunological activities of the antibody fractions against the appropriate haptens were confirmed by immunodiffusion, as shown in Fig. 9a. The protein moiety on the DDA-RSA was labeled with iodine ^{125} (Isoserve Cambridge Nuclear; carrier free) according to the technique of Hunter and Greenwood (J. Biochem., 1963, 89, 114), with minor modifications. That these tagged hapten-protein complexes retained immunological activity was confirmed by the results of immunodiffusion tests, as shown in Fig. 9b.

Experiments Aimed at Developing a Sensitive Immunoassay System with These Reagents.

Having satisfactorily confirmed that the antibodies evoked

in these animals were directed against the pesticide derivative haptens, investigations were undertaken with the goal of using these reagents as the basis for a highly sensitive detection system. For these studies, emphasis was placed on insolubilized antibody particles, since Wide and Porath have shown (op. cit.) that such methods are capable of furnishing the basis of assay techniques with sensitivities as high as 1 picogram/ml. A considerable number of antibody insolubilization procedures were studied, as detailed below, and in each instance parallel preparations were made with normal (nonimmune) rabbit immunoglobulin fractions. It was decided at the outset that these reagents would be acceptable for use in a sensitive immunoassay system only if their uptake of tagged pesticide-hapten is the result of an immune reaction and only if the uptake is restricted essentially to the insolubilized antibody preparations. As will be described, a very troublesome problem was presented by the nonspecific uptake of tagged antigen from unknown causes.

The insolubilized immunoadsorbents prepared and tested for specific uptake of DDA-RSA tagged with I^{125} are discussed below.

Sephadex G-25, superfine (particle size 10 to 40 μ). This carrier, which was used by Wide and Porath, was first treated with cyanogen bromide (Immunochem., 1967, 4, 381) and then was coupled with either antibody to DDA-RSA or with normal rabbit immunoglobulin, according to the technique described by these authors.

The particles were suspended in buffered saline solution containing 1 mg/ml of bovine serum albumin as a stabilizer. To 1 ml of each suspension, 0.1 ml of I^{125}-tagged DDA-RSA was added; the mixture was shaken thoroughly and then incubated at room temperature for 24 hours with occasional shaking. The samples were then centrifuged and twice washed thoroughly with buffered saline. The sediment was then counted in a Packard liquid scintillation counter. Typical results are shown in Table I, where it may be seen that in one test the nonspecific uptake of antigen by insolubilized normal gamma globulin was almost the same as the uptake by immune globulin.

TABLE I

Experiment	Sephadex Insolubilized Immunoglobulin	Isotope Count
1	Normal	78,500
	Anti-DDA-RSA	79,000
2	Normal	72,000
	Anti-DDA-RSA	98,000

Enzite. This cellulose derivative contains a carboxyhydrazide group covalently bound to the cellulose background, a substitution that allows protein to be chemically united to the cellulose particles. This derivative is now available from a chemical company (Gallard-Schlisinger Corp.), and samples were used to prepare insolubilized anti-DDA-RSA and normal rabbit gamma globulin.

To 1 ml of these suspensions in buffered saline was added 0.1 ml of I^{125}-labeled DDA-RSA. After incubation at room temperature for one hour with agitation, each sample was centrifuged and washed five times with buffered saline. The sediments were then counted briefly for radioactivity. Typical results are shown in Table II.

TABLE II

Experiment	Enzite Insolubilized Immunoglobulin	Isotope Count
1	Normal	173
	Anti-DDA-RSA	276
2	Normal	187
	Anti-DDA-RSA	221

Although the antibody "captured" somewhat higher levels of pesticide-albumin complex than in the Sephadex procedure, the nonspecific uptake by non-antibody immunoglobulin was excessive.

Antibody Adsorbed onto Plastic Surfaces

It is well known that most, if not all, proteins are rapidly and tenaciously adsorbed to plastic or glass surfaces. This fact has been used to advantage by Catt and Treasor (Science, 1967, 158, 1570) as the basis for a quite sensitive immunoassay system. Following their recommendation, we coated plastic scintillation vials with whole serum antibody or with normal rabbit serum (1:200 dilution with barbital

buffer, pH 9.0). After the vials were washed thoroughly with the same buffer and drained, I^{125}-tagged DDA-RSA diluted 1:20 in a 1% human plasma solution was added, and the vials were incubated for 1 hour at room temperature. The vials were then drained and washed twice with distilled water. Scintillation fluid was added, and the vials were counted in the liquid scintillation counter. There was no significant difference between specific and nonspecific uptakes.

Carboxymethyl cellulose has been utilized as a carrier for covalently bonded antigens. Uniform small particles of this substance were treated with 1-ethyl-3 (3-dimethylaminopropyl) carbodiimide HCl, a water-soluble carbodiimide coupling reagent, in the presence of the DDA-RSA antibody immunoglobulin or normal rabbit immunoglobulin, according to the procedure of Goodfriend et al. (Science, 1964, 144, 1344). Aliquots of these suspensions were mixed with samples of I^{125}-labeled DDA-RSA and were incubated at room temperature overnight with stirring. After the mixture was centrifuged and washed five times with buffered saline, the sediment was counted in the liquid scintillation counter. Typical results are shown in Table III.

TABLE III

Experiment	Immunoadsorbent prepared from Immunoglobulin	Isotope Uptake
1	Normal	387
	Anti-DDA-RSA	485
2	Normal	390
	Anti-DDA-RSA	465

Uptake was approximately 100 counts per minute greater with the antibody than with normal immunoglobulin. Since this difference is not sufficient to form the basis for a sensitive immunoassay, attempts were made to further reduce the nonspecific adsorption without affecting the immune complexes. In an effort to achieve this objective, the sediment was subjected to additional washings with tris-NaCl buffer at pH 8.0. Counts were again performed, and the values are shown in Table IV.

TABLE IV

Experiment	Immunoadsorbent prepared from Immunoglobulin	Isotope Uptake after pH 8.0 washes
1	Normal	262
	Anti-DDA-RSA	363
2	Normal	333
	Anti-DDA-RSA	398

Further attempts to elute the nonspecifically adsorbed pesticide-hapten without affecting the immune uptake were made by treating the mixture with a relatively low concentration of sodium perchlorate, 0.3 M, pH 7.4. This and other chaotropic ions are known to affect the bonds which take part in such adsorption (Dandliker et al., Biochem., 1967, 6, 1460). The residual isotope counts are shown in Table V. Even though the antibody uptake was still greater than the nonspecific adsorption, the latter had not been sufficiently reduced for the required purpose.

TABLE V

Experiment	Immunoadsorbent prepared from Immunoglobulin	Isotope Uptake after Perchlorate Treatment
1	Normal	242
	Anti-DDA-RSA	307
2	Normal	261
	Anti-DDA-RSA	301

Protapol Disc Immunoadsorbents

Discs suitable for the covalent bonding of antibody are made available by Imperial Chemicals of Australia under the name "Protapol". These units, prepared from polystyrene copolymer and a thiocyanate derivative, have been successfully employed for the isotope assay of insulin. In our tests with pesticide antibodies, the procedures described by Catt et al. (J. Clin. Endocrin. and Metab., 1968, 28, 121) were followed. Whole antibody serum and normal rabbit serum were diluted 1:200 with .05 M sodium bicarbonate buffer, pH 9.6. The discs, in scintillation vials, were soaked in these solutions on a rotator overnight. They were thoroughly washed four times with buffered saline, and then I^{125}-labeled DDA-RSA was added. The discs were allowed to stand at room temperature overnight, again with stirring. They were washed six times with tris-NaCl buffer (pH 7.6), scintillation fluid was added, and isotope counts were performed. The results, as exemplified in Table VI, revealed a high degree of nonspecific uptake, even though the immune binding was slightly greater.

TABLE VI

Experiment	Immunoadsorbent prepared from Immunoglobulin	Isotope Uptake
1	Normal	1,027
	Anti-DDA-RSA	1,125
2	Normal	927
	Anti-DDA-RSA	1,115

Further attempts were made to remove or prevent the nonspecific uptake seen in this experiment. The test was repeated as above, except that the discs were saturated with bovine serum albumin (0.5% BSA in normal rabbit serum) after the antibody had been covalently bonded to them. It was hoped that the nonspecific adsorption sites would be thoroughly saturated by such a procedure. Following this, the discs were exposed to the I^{125}-labeled DDA-RSA. Here too, however, the nonspecific uptake was almost as great as in Table VI. In a further attempt to reduce the nonspecific uptake, the experiment was run again; this time the discs were washed with 0.5 M sodium perchlorate after exposure to the tagged hapten-RSA. After nine washings with this chaotropic ion, there was still only a small difference between the specific and nonspecific uptakes.

Glutaraldehyde Insolubilization of Antibody

Avrameas and Ternynck have shown (J. Biol. Chem., 1967, 242, 1651) that cross-linked antibody molecules can be insolubilized without altering their immunological activity. Accordingly, anti-DDA

gamma globulin was insolubilized with glutaraldehyde by the simplified method of Joniau, Onkelinx, and Lontie (Int. Arch. of Physiol. & Bioch., 1968, 76, 182). The resultant gel-like particles were washed and resuspended in buffered saline. Aliquots of 0.5 ml of these suspensions were added to samples of I^{125}-tagged DDA-RSA in bovine serum albumin solution (3 mg/ml). After two hours at room temperature, the samples were centrifuged and then washed with pH 7.2 tris-NaCl buffer. The sediments were suspended in 1 ml of 2N NaOH and counted in a liquid scintillation counter. Various pH buffers were used as washes (i.e., pH 4.7 and 8.7) without causing significant differences between the specific and nonspecific uptakes. The particles were also washed with 0.5 M and 1.0 M sodium perchlorate, but the differences remained small. In addition, replicate values showed more discrepancies than were found with the other immunoadsorbents.

Other attempts were made to reduce the nonspecific adsorption of the tagged pesticide-albumin complex to insolubilized normal rabbit immunoglobulin. To several of the insolubilized preparations, a detergent (Tween 80) was added just prior to addition of the tagged DDA-RSA. This detergent has been reported to reduce such nonspecific effects in other highly sensitive isotope immunoassays. However, at several concentrations of Tween 80 (0.05, 0.1, and 0.2%) virtually all uptake was prevented by both the immune and normal insolubilized particles.

Tests with C^{14}-Labeled DDT

Since it was not possible to covalently bind DDT itself to a carrier protein to test the cross-reactivity of the native pesticide with antibodies to DDA, a sample of C^{14}-labeled DDT was obtained from Mallinckrodt for this purpose (specific activity: 3.8 millicuries/mg). An appropriate dilution (1:10,000) of this tagged pesticide in buffered saline was made, and samples were added to aliquots of glutaraldehyde anti-DDA-RSA and normal rabbit serum immunoglobulins. After incubation at room temperature for two hours with stirring, the particles were washed four times with buffered saline and then counted in the scintillation counter. No appreciable differences were observed between specific and nonspecific uptakes.

SUMMARY AND CONCLUSIONS

These investigations have completely confirmed the feasibility of producing antibodies to DDT and Malathion derivatives. For the first time in these studies, it was possible to accomplish this by immunizing animals with pesticide-protein complexes in which the protein carrier was derived from the species being immunized. Thus, DDA-rabbit serum albumin or Malathion anhydride-rabbit serum albumin conjugates were injected into rabbits in complete Freund's adjuvants at multiple intradermal sites, and antibody responses were encountered by the third or

fourth injection. By immunodiffusion, these antibodies were unequivocally demonstrated to be highly specific for each of the pesticide derivatives, and no cross-reactions were observed with appropriate antisera. In some samples, the antiserum specimens revealed reactions with the rabbit serum albumin carrier itself, or with some contaminant in the crystalline rabbit serum albumin utilized. In these cases, this contaminating antibody could be easily absorbed out to yield an antibody solution which, again, was highly specific for the pesticide derivatives used. Immunoelectrophoretic analysis with such specific anti-DDA or anti-Malathion anhydride antibodies revealed that the electrophoretic mobilities of the pesticide-albumin complexes differed from that of the native rabbit serum albumin. This alteration of mobility was most marked in the case of Malathion anhydride-rabbit serum albumin complex. Gas chromatographic analysis of a preparation of DDA-RSA demonstrates that the complex contained about 10% by weight of DDA. Final complete and conclusive demonstrations that these reactions were specific for the pesticide derivative-hapten were obtained by cross-absorption studies. Thus, anti-DDA-RSA antibodies, after thorough absorption with Malathion anhydride-RSA, fully retained their antibody activity against the DDA derivative. Reciprocally, anti-Malathion anhydride-RSA antibodies, after thorough absorption with DDA-RSA, showed no reduction in the immune precipitate with Malathion anhydride-rabbit serum albumin.

A series of other rabbits were immunized with the native

pesticides, in physical combination with rabbit serum albumin, to determine whether the parent molecules might also prove to be antigenic themselves. The same route of immunization was followed in a series of 10 rabbits, using the same dosage schedule as with the previous groups. None of these animals revealed any immune response during the entire course of the experiment.

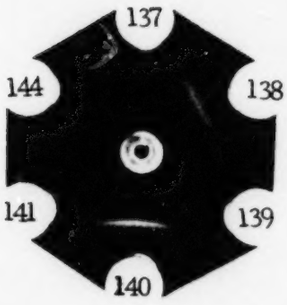
Although the reactivity of the antibodies with the pesticide derivatives is unequivocal, further sophisticated chemical work is required to elucidate the precise chemical structure of the actual pesticide derivative which is functioning as a hapten. On the basis of the purified preparations used to synthesize the conjugates, it is likely that DDA and Malathion anhydride actually represent the functional haptens, but it is conceivable that further degradation occurs in vivo, with the result that the immune response is directed against still another pesticide derivative. It is readily possible to perform studies which would more precisely identify the hapten structure and confirm its identity with the DDA or Malathion anhydride.

Immunoglobulin fractions from the various antisera active against the pesticide-albumin complexes were prepared by salting out procedures. These immunoglobulin concentrates were insolubilized by a variety of techniques which had been described and shown previously

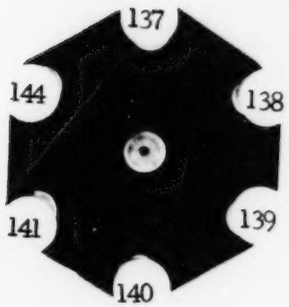
to be suitable for use in highly sensitive immunological assay techniques. The pesticide-albumin complexes were tagged in the protein moiety with I^{125} by several methods, which also had been thoroughly worked out, in connection with very sensitive hormone immunoassays. In numerous experiments with such insolubilized antibodies, uptake of the isotopically tagged DDA-RSA was shown to occur, but in most instances almost equivalent nonspecific uptake of the same tagged pesticide-protein complex took place with similarly insolubilized normal rabbit serum immunoglobulins. Numerous attempts were made and various environmental conditions were explored to prevent or minimize this nonspecific uptake, so that the "capture" of the iodine-tagged DDA-RSA by the insolubilized antibody could then serve satisfactorily as the basis for the development of a highly sensitive immunoassay system. For unknown reasons, it has not been possible to prevent this nonspecific uptake to a degree which would permit such an assay system to be developed. The reasons why this adsorption factor should prove of such magnitude in the case of antibodies to these pesticide derivatives are not clear. The situation is particularly mystifying because of their relative insignificance in hormone immunoassays, which utilize virtually identical procedures. It seems reasonable to expect that these technical difficulties can eventually be resolved by varying the conditions of insolubilization, the milieu in which the reactions occur, the type of isotope tagging utilized,

or by employing other procedures which would prevent the nonspecific uptake of the hapten-protein complex. Clarification of this problem should then allow the rapid development of an exquisitely sensitive assay system.

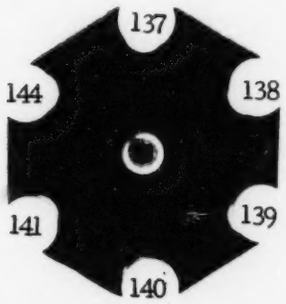
The results to date thus unequivocally demonstrate that (1) it is possible to develop specific antibodies to two pesticide derivatives, DDA and Malathion anhydride; (2) these antibodies are highly specific for each pesticide derivative; and (3) given enough time, the technical difficulties which have been encountered in utilizing these antibodies for the development of a highly sensitive immunoassay procedure could be resolved. Such a technique would offer investigators interested in pollution problems a simultaneously highly specific and highly sensitive method for detecting and measuring these pesticides, without the need for preliminary treatment of the samples being investigated.



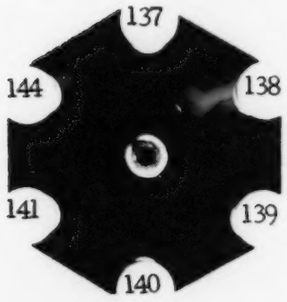
a. Central well
DDA-Anhydride-RSA
1 mg/ml



c. Central well
DDT-RSA "conjugate"
1 mg/ml



b. Central well
RSA (1 mg/ml)



d. Central well
DDT 1 mg/ml

Fig. 1. Immunodiffusion tests with several antisera to DDA-anhydride-RSA, and DDT-RSA "conjugates."
137, 138, 139, 140, 141 - antisera to DDA-anhydride-RSA from these rabbits
144, antiserum to DDT-RSA "conjugate."

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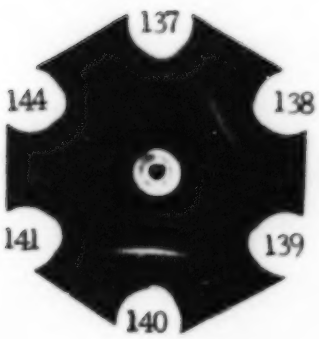
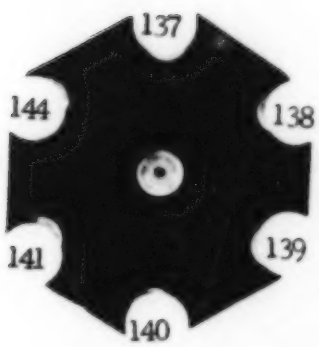
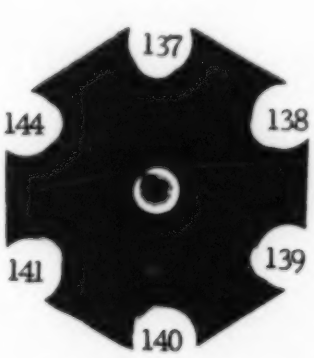
The results to date thus unequivocally demonstrate that (1) it is possible to develop specific antibodies to two pesticide derivatives, DDA and Malathion anhydride; (2) these antibodies are highly specific for each pesticide derivative; and (3) given enough time, the technical difficulties which have been encountered in utilizing these antibodies for the development of a highly sensitive immunoassay procedure could be resolved. Such a technique would offer investigators interested in pollution problems a simultaneously highly specific and highly sensitive method for detecting and measuring these pesticides, without the need for preliminary treatment of the samples being investigated.



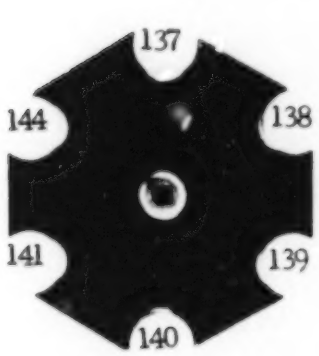
a. Central well
DDA-Anhydride-RSA
1 mg/ml



c. Central well
DDT-RSA "conjugate"
1 mg/ml

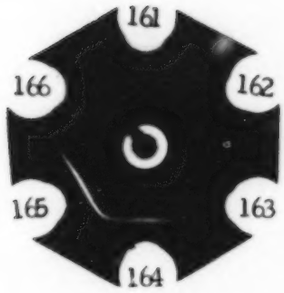


b. Central well
RSA (1 mg/ml)

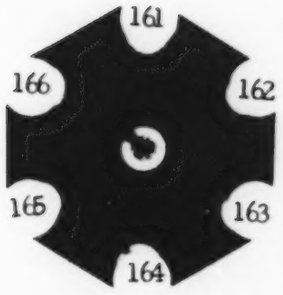


d. Central well
DDT 1 mg/ml

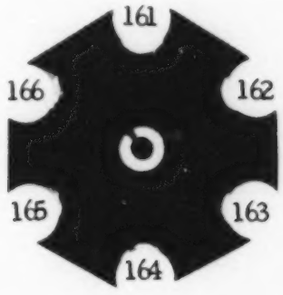
Fig. 1. Immunodiffusion tests with several antisera to DDA-anhydride-RSA, and DDT-RSA "conjugates."
137, 138, 139, 140, 141 - antisera to DDA-anhydride-RSA from these rabbits
144, antiserum to DDT-RSA "conjugate."



a. Central well
Malathion-Anhydride-RSA
1 mg/ml

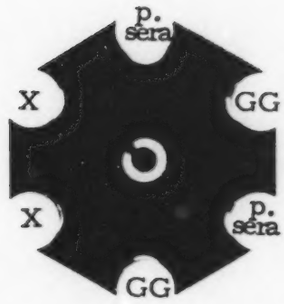


b. Central well
Native Malathion-
RSA "conjugate"
1 mg/ml



c. Central well
RSA
1 mg/ml

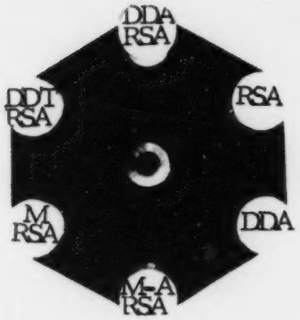
Fig. 2. Immunodiffusion reactions with antisera to malathion-anhydride-RSA complexes. The numbers refer to specimens from different rabbits.



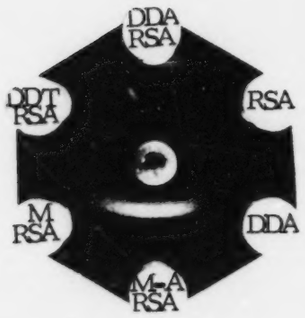
Central well
DDA-RSA (1 mg/ml)

Fig. 3. Immunodiffusion reactions with the immunoglobulin fractions of anti-DDA-RSA sera.

p. sera = pooled whole antiserum
GG = 3% immunoglobulin concentrate from this serum pool.



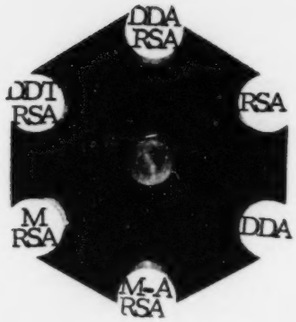
a. Central well
Anti-DDA-RSA
#137



b. Central well
Anti-malathion-anhydride-RSA
#165

Fig. 4. Lack of cross-reaction of anti-DDA-RSA and malathion-anhydride-RSA antibodies with their respective antigens (all at 1 mg/ml).

DDA-RSA: DDA rabbit serum albumin conjugate
RSA: rabbit serum albumin
DDA: DDA anhydride in normal rabbit serum
M-A RSA: malathion anhydride - rabbit serum conjugate
M RSA: native malathion - rabbit serum albumin conjugate
DDT-RSA: native DDT rabbit serum albumin conjugate

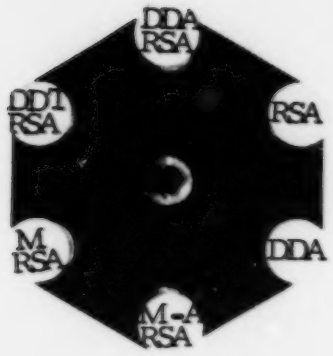


a. Central well
Anti-DDA-RSA
#138

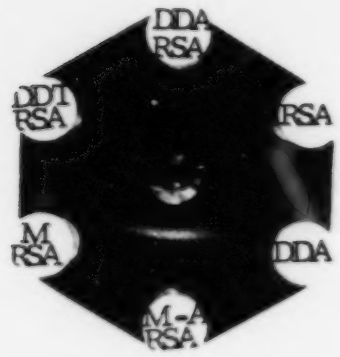
b. Central well
Anti-malathion-anhydride-RSA
#164

Fig. 5. Reactions of certain anti-DDA-RSA or anti-malathion-anhydride-RSA sera with normal rabbit serum albumin, as well as with the immunizing complexes. All antigens at 10 mg/ml.

DDA-RSA: DDA rabbit serum albumin conjugate
RSA: rabbit serum albumin
DDA: DDA anhydride in normal rabbit serum
M-A RSA: malathion anhydride - rabbit serum conjugate
M RSA: native malathion - rabbit serum albumin conjugate
DDT-RSA: native DDT rabbit serum albumin conjugate



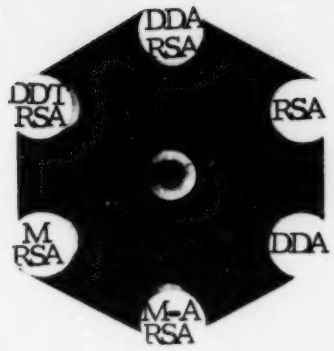
a. Central well
Anti-DDA-RSA, #138
absorbed with normal
rabbit serum albumin



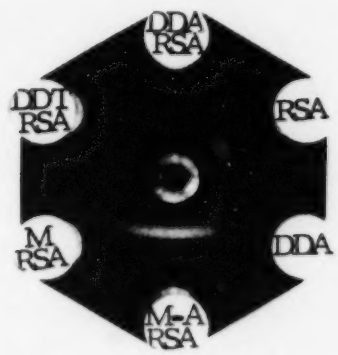
b. Central well
Anti-malathion-anhydride-RSA
#164, absorbed with normal
rabbit serum albumin

Fig. 6. Removal of non-pesticide antibodies in certain antisera by absorption with normal rabbit serum albumin (10 mg/ml of antiserum)
All test antigens at 10 mg/ml

DDA-RSA: DDA rabbit serum albumin conjugate
RSA: rabbit serum albumin
DDA: DDA anhydride in normal rabbit serum
M-A RSA: malathion anhydride - rabbit serum conjugate
M RSA: Native malathion - rabbit serum albumin conjugate
DDT-RSA: native DDT rabbit serum albumin conjugate



a. Central well
Anti-DDA-RSA absorbed with normal rabbit serum albumin and with malathion-anhydride-RSA (each 10 mg/ml)



b. Central well
Anti-malathion-anhydride-RSA absorbed with normal rabbit serum albumin and with DDA-RSA (each at 10 mg/ml)

Fig. 7. Conclusive demonstration of the hapten specificity of both DDA-RSA and malathion-anhydride-RSA antibodies, by means of cross-absorption studies. All test antigens at 10 mg/ml.

DDA-RSA: DDA rabbit serum albumin conjugate
RSA: rabbit serum albumin
DDA: DDA anhydride in normal rabbit serum
M-A RSA: malathion anhydride - rabbit serum conjugate
M RSA: native malathion - rabbit serum albumin conjugate
DDT-RSA: native DDT rabbit serum albumin conjugate

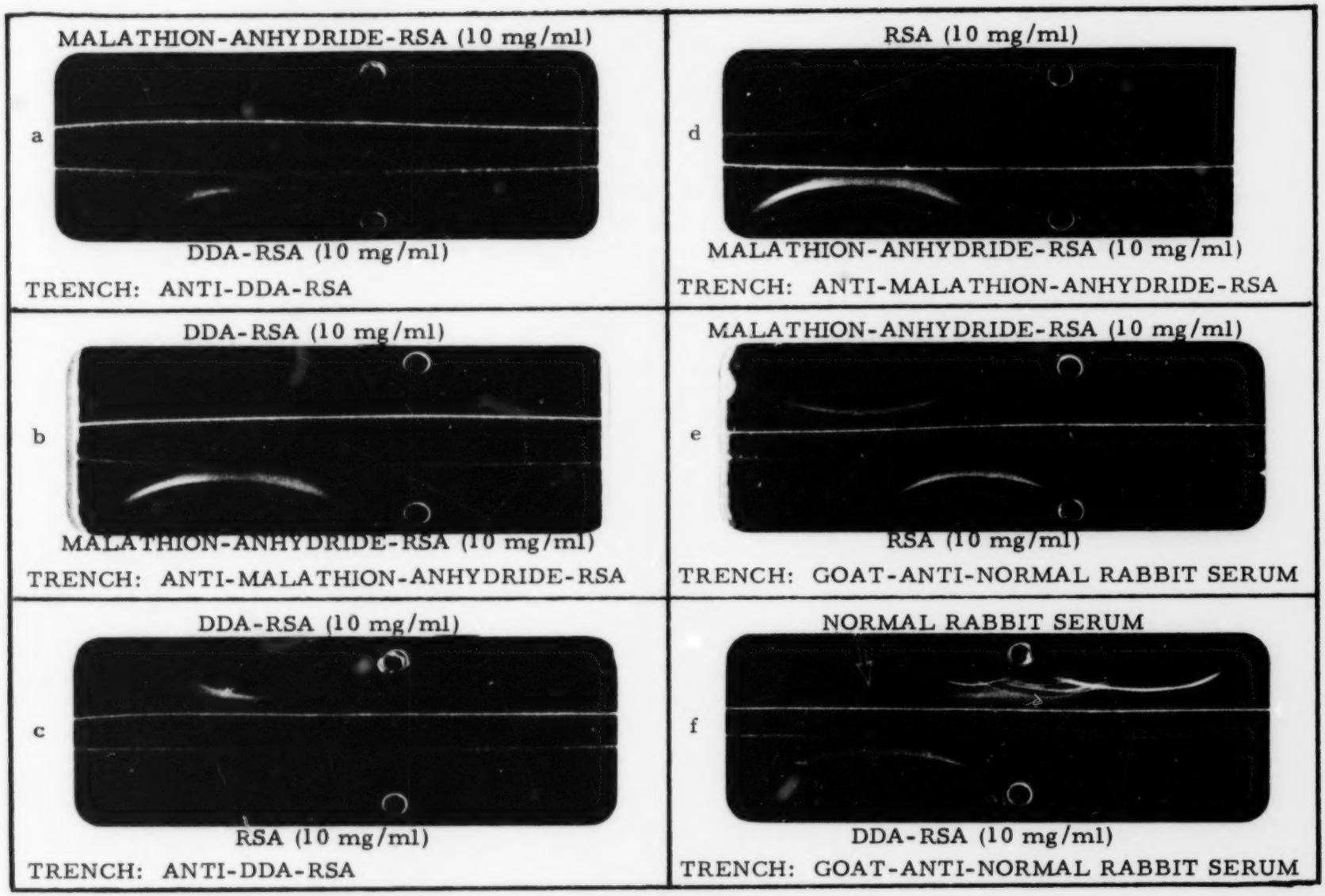


Fig. 8. Immunoelectrophoretic studies of the immune reactions between anti-pesticide antibodies and their respective antigens.

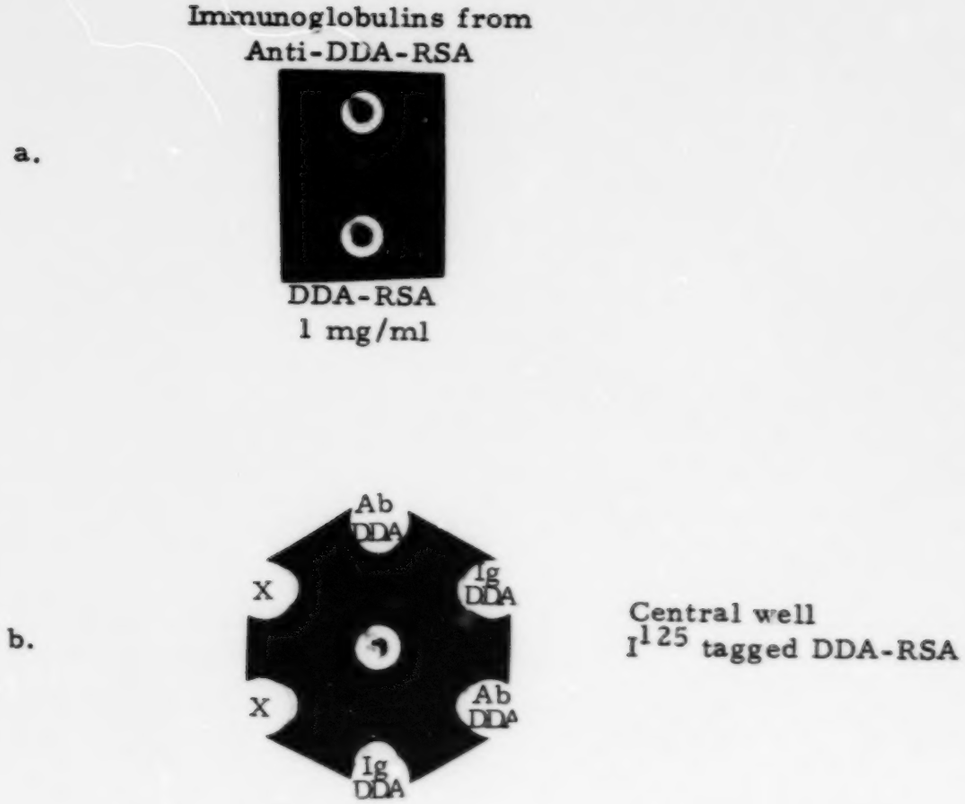


Fig. 9. Immunodiffusion reactions of immunoglobulin concentrates from antisera to DDA-RSA, and retention of reactivity in I^{125} labelled DDA-RSA.

Ab-DDA: antiserum against DDA-RSA
Ig-DDA: immunoglobulin concentrate from antiserum to DDA-RSA (3%)
X: empty